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(54) METHOD FOR DETERMINING PYROPHOSPHORIC ACID AND NUCLEIC ACID AND APPARATUS THEREFOR

(57)Abstract:

PROBLEM TO BE SOLVED: To provide a method for highly accurately detecting and determining pyrophosphoric acid, to provide a method for detecting and determining nucleic acid by various nucleic acid proliferation methods utilizing the method for detecting and determining the pyrophosphoric acid, and to provide an apparatus useful for the method.

SOLUTION: This method for accurately detecting and determining the pyrophosphoric acid is characterized by comprising a process for adding inosinic acid and/or xanthylic acid and a tetrazolium salt to a solution containing pyrophosphoric acid, a process for treating the pyrophosphoric acid with hypoxanthine phosphoribosyltransferase and dehydrogenase/oxidase to obtain the formazan, and a process for detecting and determining the produced formazan.

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CLAIMS

[Claim(s)]
[Claim 1] The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds inosinic acid and/or the xanthylic acid, and tetrazolium salt in the solution containing a pyrophosphoric acid, the process which hypoxanthine phosphoribosyltransferase, and the xanthine dehydrogenase/oxidase are made to act, and changes a pyrophosphoric acid at formazan, and providing detection and the process which carries out a quantum for the formazan to generate.
[Claim 2] The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds inosinic acid and/or the xanthylic acid in the solution containing a pyrophosphoric acid, the process which hypoxanthine phosphoribosyltransferase, and the xanthine dehydrogenase/oxidase are made to act, and changes a pyrophosphoric acid into a hydrogen peroxide or superoxide, and providing detection and the process which carries out a quantum for the hydrogen peroxide or superoxide to generate.
[Claim 3] The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds an oxal acid in the solution containing a pyrophosphoric acid, the process which phosphoenolpyruvate carboxykinase (pyrophosphoric acid) is made to act and changes a pyrophosphoric acid into a carbon dioxide, and providing detection and the process which carries out a quantum for the carbon dioxide to generate.
[Claim 4] The detection and the quantum approach of a pyrophosphoric acid characterized by including detection and the process which carries out a quantum at the sample solution containing a pyrophosphoric acid for D-phenylalanine, the process which adds adenosine diphosphate, the process which a phenylalanine racemase is made to act and changes a pyrophosphoric acid at L-phenylalanine, and the L-phenylalanine to generate.
[Claim 5] The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds adenylyl sulfate to the sample solution containing a pyrophosphoric acid, the process which an adenosine-triphosphate sulfurylase is made to act and changes a pyrophosphoric acid into sulfate ion, and including detection and the process which carries out a quantum for the sulfate ion to generate.
[Claim 6] The detection and the quantum approach of a nucleic acid characterized by including detection and the process which carries out a quantum using the approach given [the process which are detection and the quantum approach of a nucleic acid, and amplifies the nucleic acid in the sample solution, and the pyrophosphoric acid contained in the sample solution after said magnification] in any 1 term of claims 1-5.
[Claim 7] The 1st process of the heat denaturation which heats the sample solution which are detection and the quantum approach of a nucleic acid, and contains a nucleic acid, and makes a nucleic acid a single strand, The 2nd process which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained at said 1st process, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid generated at said 2nd process, carrying out an expanding reaction and compounding a DNA complementary strand Detection and the quantum approach of a nucleic acid of providing detection and the 4th process which carries out a quantum using the approach of five claim 1 thru/or given in any 1 term for the

pyrophosphoric acid which separated at the process of the 3rd expanding reaction which separates a pyrophosphoric acid, and said 3rd process.

[Claim 8] The detection and the quantum approach of a nucleic acid according to claim 7 characterized by performing the expanding reaction in said 3rd process in the reactor which fixed DNA polymerase, and performing conversion of the pyrophosphoric acid in said 4th process in the reactor which fixed the enzyme.

[Claim 9] The 1st process of the heat denaturation which heats a nucleic acid, adenylyl sulfate, and the sample solution containing luciferin, and makes a nucleic acid a single strand, The 2nd process which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained at said 1st process, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid generated at said 2nd process, carrying out an expanding reaction and compounding a DNA complementary strand To the pyrophosphoric acid which separated at the process of the 3rd expanding reaction which separates a pyrophosphoric acid, and said 3rd process They are detection and the quantum approach of a nucleic acid of providing detection and the 4th process which carries out a quantum for light as matter which the adenosine-triphosphate sulfurylase and luciferase which are an enzyme are made acting, and is generated. The detection and the quantum approach of a nucleic acid characterized by performing the expanding reaction in said 3rd process in the reactor which fixed DNA polymerase, and performing conversion of the pyrophosphoric acid in said 4th process in the reactor which fixed said enzyme.

[Claim 10] Detection and the quantum approach of the nucleic acid of claim 7-9 given in any 1 term characterized by for said 1st, 2nd, 3rd, and 4th processes carrying out multiple-times circulation one by one, and performing them when the sample solution returns.

[Claim 11] The 1st processing section which heats the sample solution containing a nucleic acid and makes a nucleic acid a single strand, The 2nd processing section which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained in said 1st processing section, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid obtained in said 2nd processing section, carrying out an expanding reaction and compounding a DNA complementary strand The 3rd processing section which separates a pyrophosphoric acid, and the 4th processing section which the enzyme of five claim 1 thru/or given in any 1 term is made to act on the pyrophosphoric acid which separated in said 3rd processing section, and changes this pyrophosphoric acid, Equipment for carrying out a nucleic acid detection and a quantum equipped with detection and the 5th processing section which carries out a quantum for the product obtained in said 4th processing section.

[Claim 12] Equipment for carrying out a nucleic acid according to claim 11 detection and a quantum characterized by having the reactor with which said 3rd processing section fixed DNA polymerase, and having the reactor with which said 4th processing section fixed said enzyme.

[Claim 13] Equipment for carrying out a nucleic acid according to claim 11 or 12 detection and a quantum characterized by having passage for the sample solution carrying out multiple-times circulation of said 1st thru/or 5th processing section one by one.

[Claim 14] The reagent kit which crawls on to detection and the quantum approach of a pyrophosphoric acid according to claim 1 and which is included in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively a pyrophosphoric acid, inosinic acid and/or the xanthylic acid, tetrazolium salt, hypoxanthine phosphoribosyltransferase, and the xanthine dehydrogenase/oxidase independently.

[Claim 15] The reagent kit which crawls on to detection and the quantum approach of a pyrophosphoric acid according to claim 2 and which is included in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively the reagent which detects a pyrophosphoric acid, inosinic acid and/or the xanthylic acid, hypoxanthine phosphoribosyltransferase, the xanthine dehydrogenase/oxidase, the hydrogen peroxide to generate, or superoxide independently.

[Claim 16] The reagent kit which crawls on detection or the reagent which carries out a quantum to detection and the quantum approach of a pyrophosphoric acid according to claim 3, and contains it in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains

respectively a pyrophosphoric acid, an oxalacetic acid, phosphoenolpyruvate carboxykinase, and the carbon dioxide to generate independently.

[Claim 17] The reagent kit which crawls on detection or the reagent which carries out a quantum to detection and the quantum approach of a pyrophosphoric acid according to claim 4, and contains it in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively a pyrophosphoric acid, D-phenylalanine, adenosine diphosphate, a phenylalanine racemase, and the L-phenylalanine to generate independently.

[Claim 18] The reagent kit which crawls on detection or the reagent which carries out a quantum, and contains it as a gap or two or more sorts of mixed reagents as a reagent which is a reagent kit useful to detection and the quantum approach of a pyrophosphoric acid according to claim 5, and contains respectively a pyrophosphoric acid, adenylyl sulfate, an adenosine-triphosphate sulfurylase, and the sulfate ion to generate independently.

[Claim 19] The reagent kit which is a reagent kit useful in detection and the quantum of the nucleic acid made into a target, and contains a reagent required in order to amplify a nucleic acid further in addition to the reagent kit of 11 claim 7 thru/or given in any 1 term.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to detection and the quantum approach of a pyrophosphoric acid. This inventions are detection and the quantum approach of a nucleic acid of having used detection and the quantum approach of this pyrophosphoric acid, and relate a nucleic acid to detection and the approach of carrying out a quantum detection and by carrying out a quantum again in the pyrophosphoric acid generated when a nuclide acid field is amplified using the various magnification approaches which include polymerase chain reaction in detail.
[0002]
[Description of the Prior Art] In fields, such as a clinical laboratory test of the infectious disease by the virus, bacteria, etc., or food poisoning inspection, it is necessary to carry out the microorganism nucleic acid of minute amounts, such as an organization, body fluid, and facilities, detection and a quantum. Moreover, in various areas of research, carrying out the quantum of the specific amount of messenger RNA manifestations discovered in an operation of various illnesses, drugs, etc. is called for.
[0003] The detection approach of the nucleic acid by the antigen detection approach or DNA probe which used the antigen-antibody reaction as an approach of identifying a pathogen etc. quickly is also performed.
[0004] However, in the antigen detection using an antigen-antibody reaction, when the antigen part of a pathogen is concealed, there is a possibility that a pathogen may be undetectable. Moreover, it is not necessarily easy to have very many antigen parts in bacteria etc., and to find out a specific common antigen part peculiar to a specific bacteria kind. Moreover, the bacteria or cell of a certain amount of number or concentration is required for detection, when the numbers of cells etc. run short, a pathogen may be unable to be detected and there is a problem also in sensibility.
[0005] On the other hand, in the nucleic-acid detection approach by the DNA probe, it is comparatively easy to find out the nucleic-acid field of a living thing kind proper, and it has the advantage that it can use as a nucleic acid probe, by carrying out cloning of this nucleic-acid field, or compounding it. However, when there are few amounts of nucleic acids used as mold also about a DNA probe method, sufficient detection sensitivity is not obtained.
[0006] Moreover, the method (the polymerase chain reaction method, PCR method) of detecting a nucleic acid for having used the approach of making a specific base sequence amplifying by DNA polymerase is used widely as an effective means. This PCR method amplifies the nuclide acid field in a nucleic-acid molecule also 1 million times within a test tube. It is also possible by using this PCR to become possible to detect, where the nuclide acid field of a target-nucleus acid is amplified, and to detect a pathogen from the nucleic acid of one molecule in sensibility. The detailed explanation about PCR is indicated by U.S. Pat. No. 4,683,195, 4,683,202, and the 4,965,188 specification.
[0007] And as the general detection approach of this amplified nuclide acid field, after staining is carried out and the approach of distinguishing in the magnitude (molecular weight) of a band which separated the reaction solution after PCR termination by agarose electrophoresis, and the method of detecting said reaction solution by the dot hybridization method are performed. Since this detection approach amplifies DNA in bacteria directly and can detect it, inspection time amount has the description that it is short and bacterial identification is easy. However, it is complicated and the electrophoretic process which time

amount requires has many inconvenient points about use in a site.
[0008] The approach (JP,5-237000,A) of measuring the amount of magnification DNA can be mentioned by adding the reagent which emits fluorescence by making an PCR product into detection and the approach of carrying out a quantum, only when it intercalates in the reaction mixture before PCR initiation with DNA beforehand, and measuring fluorescence intensity using a spectrophotofluorometer, without performing electrophoresis. The amount of initial mold of a target-nucleus acid can perform PCR under existence of an intercalator nature fluorochrome, can measure the fluorescence of reaction mixture for every PCR cycle, and can search for it from the change. However, it has the fault that the equipment for measuring the fluorescence intensity designed for such a purpose for every cycle is expensive. Moreover, DNA and the reagent to intercalate are harmful for a living body.
[0009] As an approach of not using an intercalation reagent with DNA, the pyrophosphoric acid of the by-product generated in the nucleic-acid magnification process by PCR is led to an adenosine triphosphate in an operation of an adenosine-triphosphate sulfurylase, an adenosine triphosphate is made to emit light to (1) WO 92/16654 or J.Immunol.Methods 156, and 55 (1992) by the luciferin-luciferase method further, and the method of detecting a pyrophosphoric acid is used for them by detecting.
[0010] Moreover, the method of detecting the gene nucleic acid made into a target is indicated by (2) JP,7-596007,A by detecting the phosphoric acid which is the product which decomposed the pyrophosphoric acid using the inorganic pyrophosphatase etc.
[0011] The present PCR reactor (the equipment which equipped an PCR reaction and coincidence with the means which measures fluorescence intensity is included) is a temperature-circulation system for pouring distributively beforehand a heat stable enzyme and a primer, and all other reagents required for an PCR reaction to a well-closed container, and repeating three reactions of the complementary strand composition by annealing and nucleic-acid polymerase of dissociation of a two nucleic-acids chain, a single-strand nucleic acid, and a primer. The target template DNA of a sample is amplified for a short time, without being polluted by the nucleic acid of other specimens by using this equipment.
[0012]
[Problem(s) to be Solved by the Invention] The measuring method (1) of the conventional pyrophosphoric acid needs an expensive chemiluminescence detector, and has the fault of luminescence which generated being unstable and losing luminescence in an instant. Therefore, it is inconvenient to measure the amount of pyrophosphoric-acid magnification generated by the various nucleic-acid amplifying methods on real time. In order to guide and detect a pyrophosphoric acid to an adenosine triphosphate furthermore, possibility that the adenosine triphosphate which exists in the living body will be detected as the background is high. Moreover, by the measuring method (2) of a pyrophosphoric acid, it has a fault, like by the well-known approach, the quantum of the phosphoric acid which is the decomposition product of a pyrophosphoric acid has low detection sensitivity, and is accompanied by processing of heavy-metal-ion waste fluid, such as molybdenum acid chloride. Since the phosphate that to a living body, food, a culture medium, etc. made to use is detected as the background, it is not suitable for practical use.
[still more]
[0013] Moreover, at the PCR reaction by the conventional temperature-circulation system, when RNA and a single stranded DNA are used as mold, also although it becomes empty and is called a thermostable enzyme, neither a lifting nor deactivation by heat can be avoided for a nonspecific magnification reaction. Moreover, it is known that the pyrophosphoric acid generated as a result of magnification will check a magnification reaction.
[0014] This invention is originated in view of the above troubles, and it aims at offering useful equipment to the detection, the quantum approach, and this approach of a nucleic acid by the various nucleic-acid amplifying methods for having used offering detection and the quantum approach of a highly precise pyrophosphoric acid, and detection and the quantum approach of this pyrophosphoric acid.
[0015]
[Means for Solving the Problem] It came [changed the pyrophosphoric acid into the matter detectable to high sensitivity by the well-known approach, using a specific enzyme, as a result of inquiring wholeheartedly that this invention persons should solve the above-mentioned technical problem, and] to complete [this] a header and this invention for being stability and it being very useful in a pyrophosphoric acid with high precision in detection of detection and the nucleic acid can carry out a quantum and according [detection of this pyrophosphoric acid and the quantum approach] to the

various nucleic-acids amplifying method further, and a quantum detection and by carrying out a quantum. Furthermore, this invention person etc. came to complete header this invention also for the above-mentioned fault at the time of performing an PCR reaction using a temperature-circulation system being solved by using the equipment using a sample-ring current method using a specific enzyme.

[0016] That is, this invention has the following configuration.

(1) The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds inosinic acid and/or the xanthylic acid, and tetrazolium salt in the solution containing a pyrophosphoric acid, the process which hypoxanthine phosphoribosyltransferase, and the xanthine dehydrogenase/oxidase are made to act, and changes a pyrophosphoric acid at formazan, and providing detection and the process which carries out a quantum for the formazan to generate.

[0017] (2) The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds inosinic acid and/or the xanthylic acid in the solution containing a pyrophosphoric acid, the process which hypoxanthine phosphoribosyltransferase, and the xanthine dehydrogenase/oxidase are made to act, and changes a pyrophosphoric acid into a hydrogen peroxide or superoxide, and providing detection and the process which carries out a quantum for the hydrogen peroxide or superoxide to generate.

[0018] (3) The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds an oxal acid in the solution containing a pyrophosphoric acid, the process which phosphoenolpyruvate carboxykinase (pyrophosphoric acid) is made to act and changes a pyrophosphoric acid into a carbon dioxide, and providing detection and the process which carries out a quantum for the carbon dioxide to generate.

[0019] (4) The detection and the quantum approach of a pyrophosphoric acid characterized by including detection and the process which carries out a quantum at the sample solution containing a pyrophosphoric acid for D-phenylalanine, the process which adds adenosine diphosphate, the process which a phenylalanine racemase is made to act and changes a pyrophosphoric acid at L-phenylalanine, and the L-phenylalanine to generate.

[0020] (5) The detection and the quantum approach of a pyrophosphoric acid characterized by the process which adds adenylyl sulfate to the sample solution containing a pyrophosphoric acid, the process which an adenosine-triphosphate sulfurylase is made to act and changes a pyrophosphoric acid into sulfate ion, and including detection and the process which carries out a quantum for the sulfate ion to generate.

[0021] (6) The detection and the quantum approach of a nucleic acid characterized by including detection and the process which carries out a quantum using the approach given [the process which are detection and the quantum approach of a nucleic acid, and amplifies the nucleic acid in the sample solution, and the pyrophosphoric acid contained in the sample solution after said magnification] in any 1 term of (1) - (5).

[0022] (7) The 1st process of the heat denaturation which heats the sample solution which are detection and the quantum approach of a nucleic acid, and contains a nucleic acid, and makes a nucleic acid a single strand, The 2nd process which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained at said 1st process, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid generated at said 2nd process, carrying out an expanding reaction and compounding a DNA complementary strand Detection and the quantum approach of a nucleic acid of providing detection and the 4th process which carries out a quantum using the approach of (1) thru/or (5) given in any 1 term for the pyrophosphoric acid which separated at the process of the 3rd expanding reaction which separates a pyrophosphoric acid, and said 3rd process.

[0023] (8) Detection and the quantum approach of a nucleic acid given in (7) characterized by performing the expanding reaction in said 3rd process in the reactor which fixed DNA polymerase, and performing conversion of the pyrophosphoric acid in said 4th process in the reactor which fixed the enzyme.

[0024] (9) The 1st process of the heat denaturation which heats a nucleic acid, adenylyl sulfate, and the sample solution containing luciferin, and makes a nucleic acid a single strand, The 2nd process which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained

at said 1st process, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid generated at said 2nd process, carrying out an expanding reaction and compounding a DNA complementary strand To the pyrophosphoric acid which separated at the process of the 3rd expanding reaction which separates a pyrophosphoric acid, and said 3rd process They are detection and the quantum approach of a nucleic acid of providing detection and the 4th process which carries out a quantum for light as matter which the adenosine-triphosphate sulfurylase and luciferase which are an enzyme are made acting, and is generated. The detection and the quantum approach of a nucleic acid characterized by performing the expanding reaction in said 3rd process in the reactor which fixed DNA polymerase, and performing conversion of the pyrophosphoric acid in said 4th process in the reactor which fixed said enzyme.

[0025] (10) Detection and the quantum approach of the nucleic acid of (7) - (9) characterized by for said 1st, 2nd, 3rd, and 4th processes carrying out multiple-times circulation one by one, and performing them when the sample solution returns given in any 1 term.

[0026] (11) The 1st processing section which heats the sample solution containing a nucleic acid and makes a nucleic acid a single strand, The 2nd processing section which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained in said 1st processing section, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid obtained in said 2nd processing section, carrying out an expanding reaction and compounding a DNA complementary strand The 3rd processing section which separates a pyrophosphoric acid, and the 4th processing section which the enzyme of (1) thru/or (5) given in any 1 term is made to act on the pyrophosphoric acid which separated in said 3rd processing section, and changes this pyrophosphoric acid, Equipment for carrying out a nucleic acid detection and a quantum equipped with detection and the 5th processing section which carries out a quantum for the product obtained in said 4th processing section.

[0027] (12) Equipment for making the nucleic acid of a publication (11) detection and a quantum characterized by having the reactor with which said 3rd processing section fixed DNA polymerase, and having the reactor with which said 4th processing section fixed said enzyme.

[0028] (13) Equipment for making the nucleic acid of a publication (11) or (12) detection and a quantum characterized by having passage for the sample solution carrying out multiple-times circulation of said 1st thru/or 5th processing section one by one.

[0029] (14) The reagent kit which crawls on to detection and the quantum approach of a pyrophosphoric acid given in (1) and which is included in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively a pyrophosphoric acid, inosinic acid and/or the xanthylic acid, tetrazolium salt, hypoxanthine phosphoribosyltransferase, and the xanthine dehydrogenase/oxidase independently.

[0030] (15) The reagent kit which crawls on to detection and the quantum approach of a pyrophosphoric acid given in (2) and which is included in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively the reagent which detects a pyrophosphoric acid, inosinic acid and/or the xanthylic acid, hypoxanthine phosphoribosyltransferase, the xanthine dehydrogenase/oxidase, the hydrogen peroxide to generate, or superoxide independently.

[0031] (16) The reagent kit which crawls on detection or the reagent which carries out a quantum to detection and the quantum approach of a pyrophosphoric acid given in (3), and contains it in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively a pyrophosphoric acid, an oxalacetic acid, phosphoenolpyruvate carboxykinase, and the carbon dioxide to generate independently.

[0032] (17) The reagent kit which crawls on detection or the reagent which carries out a quantum to detection and the quantum approach of a pyrophosphoric acid given in (4), and contains it in it as a gap or two or more sorts of mixed reagents as a reagent which is a useful reagent kit and contains respectively a pyrophosphoric acid, D-phenylalanine, adenosine diphosphate, a phenylalanine racemase, and the L-phenylalanine to generate independently.

[0033] (18) The reagent kit which crawls on detection or the reagent which carries out a quantum, and contains it as a gap or two or more sorts of mixed reagents as a reagent which is a reagent kit useful to detection and the quantum approach of a pyrophosphoric acid given in (5), and contains respectively a

pyrophosphoric acid, adenylyl sulfate, an adenosine-triphosphate sulfurylase, and the sulfate ion to generate independently.
[0034] (19) The reagent kit which is a reagent kit useful in detection and the quantum of the nucleic acid made into a target, and contains a reagent required in order to amplify a nucleic acid further in addition to the reagent kit of (7) - (11) given in any 1 term.
[0035]
[Embodiment of the Invention] Hereafter, this invention is explained to a detail.
[0036] Detection and the quantum approach of the pyrophosphoric acid offered by this invention are developed as a part as which this invention persons develop the measuring method of the pyrophosphoric acid generated by the various nucleic-acid magnification technique. therefore, the PCR method and Isothermal and Chimeric primer-initiated Amplification of Nucleic acids -- law (ICAN law) and loop-mediated isothermal amplification -- it is suitable as a measuring method of the pyrophosphoric acid in the case of carrying out which will presume change of the amount of nucleic acids amplified using various technique, such as law (LAMP law), from the amount of pyrophosphoric acids. However, the pyrophosphoric-acid measuring method of this invention is effective also as a measuring method of the various pyrophosphates added by food as a food additive only as detection and the quantum approach of the pyrophosphoric acid generated by the nucleic-acid amplifying method.
[0037] In addition, as matter which can be measured using the approach of this invention, inosinic acid, adenosine diphosphate, an oxalacetic acid, D-phenylalanine, adenylyl sulfate, etc. can be mentioned.
[0038] After making an adenosine-triphosphate sulfurylase act on a pyrophosphoric acid conventionally as a pyrophosphoric-acid measuring method as mentioned above, the approach of detecting luminescence to generate, and the method of detecting the phosphoric acid which is the product which decomposed the pyrophosphoric acid using the inorganic pyrophosphatase etc. are used using the luciferin-luciferase method. By this invention, it newly finds out detection and that a quantum can be carried out for a pyrophosphoric acid to stability and high degree of accuracy by combining a specific enzyme and a specific reagent so that it may explain in full detail below.
[0039] The 1st approach for measuring the pyrophosphoric acid newly offered by this invention adds the inosinic acid of an initial complement or the xanthylic acid, and tetrazolium salt to the test portion containing a pyrophosphoric acid, makes hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), and the xanthine dehydrogenase (EC 1.1.1.204)/oxidase (EC 1.1.3.22) act, and measures the formazan to generate. This measuring method becomes possible [carrying out the quantum of the pyrophosphoric acid with high precision almost quantitatively, when two-mol formazan generates from one mol of pyrophosphoric acids when inosinic acid is used, it uses that one-mol formazan generates and it is developed from one mol of pyrophosphoric acids, when the xanthylic acid is used, and a quantum carries out the quantum of the easy formazan by the well-known approach if an enzyme reaction is performed under existence of superfluous inosinic acid or the superfluous xanthylic acid, and superfluous tetrazolium salt]. As an example of the measuring method of formazan, since coloration is carried out, the quantum of the formazan can be carried out by measuring an absorbance with a spectrophotometer. Both inosinic acid and the xanthylic acid can also be used for coincidence by this measuring method. The above-mentioned reaction at the time of using inosinic acid is shown by the following reaction formulae.
[0040]
[Formula 1]

ヒポキサンチンホスホリボシルトランスフェラーゼ
ピロリン酸＋イノシン酸 ⟶ ヒポキサンチン＋ＰＲＰＰ
（ＰＲＰＰ：ホスホリボシルピロフォスフェート）

キサンチンデヒドロゲナーゼ／オキシダーゼ
ヒポキサンチン＋２テトラゾリウム塩 ⟶ ２ホルマザン＋尿素

[0041] The addition sequence of each reagent and an enzyme may add not the place to ask but any first, and may add these as mixture.
[0042] Especially the tetrazolium salt used for this approach is not restricted, and can mention PARAYODO nitro tetrazolium violet, thiazoyl blue, neotetrazolium chloride, a nitroblue tetrazolium, a

tetra-nitroblue tetrazolium, tetrazolium blue, tetrazolium red, tetrazolium violet, a thio carbamoyl nitroblue tetrazolium, triphenyl tetrazolium chloride, etc.

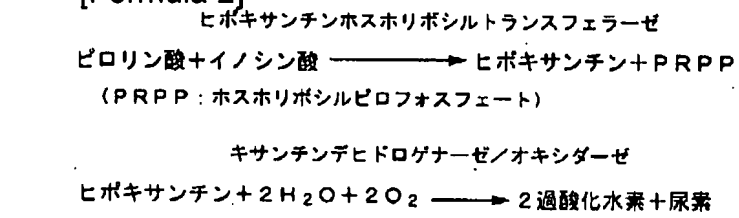
[0043] for example, in measuring a pyrophosphoric acid using PARAYODO nitro tetrazolium violet Usually, inosinic acid of 0.1 - 100mM extent, PARAYODO nitro tetrazolium violet of 0.1 - 100mM extent, The potassium chloride of 1 - 1000mM extent, the magnesium chloride of 1 - 1000mM extent, The hypoxanthine phosphoribosyltransferase of 50 - 5000 unit/L extent, The tris hydrochloric-acid buffer solution (preferably about 6.5 to 8.0 pH) of 5 containing the xanthine dehydrogenase/oxidase of 5 - 500 unit/L extent - 500mM extent is used. What is necessary is just to carry out the colorimetry of the formazan to generate on the wavelength of about 547nm, after making it react at 25-37 degrees C for 10 to 120 minutes.

[0044] The 2nd approach adds the inosinic acid or the xanthylic acid of an initial complement to the test portion containing a pyrophosphoric acid, makes hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), and the xanthine dehydrogenase (EC 1.1.1.204)/oxidase (EC 1.1.3.22) act, and measures the hydrogen peroxide or superoxide to generate.

[0045] Both inosinic acid and the xanthylic acid can also be used for coincidence by this measuring method. When any of inosinic acid and the xanthylic acid are used as a substrate, superoxide (O2) once generates, but since the superoxide generated by making it coexist with suitable chemiluminescence matter (for example, luminol etc.) can be led to the direct luminescence kind instead of a hydrogen peroxide, the amount of pyrophosphoric acids can be measured by measuring chemiluminescence reinforcement. On the other hand, in not using a chemiluminescence reagent, it changes superoxide into a hydrogen peroxide whether you are Sumiya. The reaction in that case can be expressed with the following reaction formulae, and as shown in the following reaction formula, the amount of hydrogen peroxides and the amount of pyrophosphoric acids which are measured have functionality, and can measure the amount of pyrophosphoric acids.

[0046]

[Formula 2]



[0047] The addition sequence of each reagent and an enzyme may add not the place to ask but any first, and may add these as mixture.

[0048] In measuring a pyrophosphoric acid using the 2nd approach Usually, inosinic acid of 0.1 - 100mM extent, oxygen of 0.1 - 100mM extent, The potassium chloride of 1 - 1000mM extent, the magnesium chloride of 1 - 1000mM extent, The hypoxanthine phosphoribosyltransferase of 50 - 5000 unit/L extent, The tris hydrochloric-acid buffer solution (preferably about 6.5 to 8.0 pH) of 5 containing the xanthine dehydrogenase/oxidase of 5 - 500 unit/L extent - 500mM extent is used. The quantum of the pyrophosphoric acid can be interrelatively carried out by making it react at 25-37 degrees C for 10 to 120 minutes, and measuring the hydrogen peroxide to generate by the well-known approach.

[0049] By the conventional pyrophosphoric-acid measuring method, since satisfying detection sensitivity was not obtained, this approach is developed for the purpose of raising the detection sensitivity of a pyrophosphoric acid by guiding and detecting to the high hydrogen peroxide of detection sensitivity with a conventional method. The advantage of guiding to a hydrogen peroxide has high detection sensitivity, and the measurement of spectrophotometer for ultraviolet and visible region is cheap, and it is mentioned that either of high sensitivity equipments, such as high equipment of versatility or a chemiluminescence detector, and a fluorescence detector, can be measured and that a hydrogen peroxide is stability in a solution.

[0050] About detection of a hydrogen peroxide, it is detectable using many conventional methods, such as the detection approach using a reagent. As the easiest mode, the approach of detecting a hydrogen peroxide using the reagent which offers a color signal according to one reaction or more with a hydrogen peroxide can be used by this measuring method. One of such approaches evaluates by wavelength of

640nm the blue coloration generated under existence of reduction type patent blue and a peroxidase.
[0051] The 3rd approach is the approach of measuring the carbon dioxide which add the oxalacetic acid of an initial complement to the test portion containing a pyrophosphoric acid, and phosphoenolpyruvate carboxykinase (pyrophosphoric acid) (EC 4.1.1.38) is made to act on it, and is generated. This reaction is shown by the following reaction formulae.
[0052]
[Formula 3]

ホスホエノールピルビン酸カルボキシキナーゼ（ピロリン酸）

ピロリン酸＋オキサロ酢酸 ⟶

ホスホエノールピルビン酸＋二酸化炭素＋リン酸

[0053] The addition sequence of the above-mentioned reagent and an enzyme may add not the place to ask but any first, and may add these as mixture.
[0054] What is necessary is just to make it react at 25-37 degrees C for 10 to 120 minutes using the tris hydrochloric-acid buffer solution (preferably about 6.5 to 9.0 pH) of 5 containing the oxalacetic acid of 0.1 - 1000mM extent, the magnesium chloride of 1 - 1000mM extent, and about [0.01-5 unit/ml] phosphoenolpyruvate carboxykinase (pyrophosphoric acid) - 500mM extent, when it is going to measure a pyrophosphoric acid. A pyrophosphoric acid can be measured by measuring the amount of generation of the carbon dioxide to generate. A well-known approach can be used as a measuring method of a carbon dioxide.
[0055] The 4th approach is an approach of making a phenylalanine racemase (EC5.1.1.11) acting on the test portion containing a pyrophosphoric acid. Add D-phenylalanine and adenosine diphosphate to the test portion containing a pyrophosphoric acid, a phenylalanine racemase (EC 5.1.1.11) is made to act on it, and the L-phenylalanine to generate is measured. This reaction is shown by the following reaction formulae.
[0056]
[Formula 4]

フェニルアラニンラセマーゼ

D-フェニルアラニン＋アデノシンニリン酸＋ピロリン酸 ⟶

L-フェニルアラニン＋アデノシン三リン酸

[0057] The addition sequence of each reagent and an enzyme may add not the place to ask but any first, and may add these as mixture.
[0058] What is necessary is for a phenylalanine racemase to be required as D-phenylalanine, adenosine diphosphate, and an enzyme as a substrate, and just to make it react at 25-37 degrees C for 10 to 120 minutes using the tris hydrochloric-acid buffer solution (about 6.5 to 8.0 pH) of 5 which usually contains the adenosine diphosphate of D-phenylalanine of 0.1 - 1000mM extent, and 0.1 - 1000mM extent, and an about [0.01-5 unit/ml] phenylalanine racemase - 500mM extent. By measuring using a rotatory-polarization detector, the amount of generation of the L-phenylalanine to generate can measure a pyrophosphoric acid.
[0059] The 5th approach is an approach of making an adenosine-triphosphate sulfurylase (EC 2.7.7.4) acting on the test portion containing a pyrophosphoric acid. Add adenylyl sulfate as a substrate to the test portion containing a pyrophosphoric acid, an adenosine-triphosphate sulfurylase (EC 2.7.7.4) is made to act, and the sulfate ion to generate is measured.
[0060]
[Formula 5]

アデノシン三リン酸スルフリラーゼ

アデニリル硫酸＋ピロリン酸 ⟶ アデノシン三リン酸＋SO_4^{2-}

[0061] The addition sequence of each reagent and an enzyme may add not the place to ask but any first, and may add these as mixture.
[0062] When it is going to measure a pyrophosphoric acid, usually The adenylyl sulfate of 0.1 - 1000mM extent, The ethylene-diamine-tetraacetic acid of 0.1 - 100mM extent, about 0.01 - 1% of cow serum albumin, The magnesium acetate of 1 - 100mM extent, the dithiothreitol of 0.001 - 1mM extent, The tris

acetic-acid buffer solution (about 6.5 to 8.0 pH) of 5 containing an about [0.01-5 unit/ml] adenosine-triphosphate sulfurylase - 500mM extent is used. The quantum of the pyrophosphoric acid can be carried out by making it react for 1 to 50 minutes, and measuring the sulfate ion to generate using a well-known approach at 25-37 degrees C. For example, a pyrophosphoric acid can be measured by adding hydrochloric-acid barium for the sulfate ion to generate under hydrochloric-acid acidity, making it react, and measuring muddiness of the generated barium sulfate using a nephelometer.

[0063] As for the reagent and enzyme which are used in the pyrophosphoric-acid measuring method of this invention, being supplied as a component of a reagent kit is advantageous. As for the component of such a reagent kit, it is desirable to be provided by suitable concentration according to a predetermined approach etc., and to be beforehand poured distributively by the container with a lid. The component contained in the reagent kit offered by this invention is the mixture of the thing which contains independently the reagent needed according to a test portion, a measuring method, etc., and the enzymes of each, two or more sorts of reagents, or an enzyme. These component slack reagent and an enzyme may be suitably packed from a viewpoint of required safety and the simplicity of handling (dry type or wet). It is also possible to get to know near concentration by the shade of the coloration by a shade comparison or a photograph, a CCD camera, etc. with the color sample by the naked eye or fluorescence, and luminescence as a simple judging for checking the existence of a pyrophosphoric acid other than the precision quantum using advanced optical instruments, such as spectrophotometer for ultraviolet and visible region, a spectrophotofluorometer, and a chemiluminescence detector, as a means to detect a pyrophosphoric acid, using this reagent kit. By time amount change of the color data based on a personal computer etc., it can use also for the quantum of a bacillus from near concentration and its time amount change.

[0064] Detection and the quantum approach of the pyrophosphoric acid of this invention are very useful in detection and the quantum of the nucleic acid by the various nucleic-acid amplifying methods. namely, PCR -- of course -- LAMP -- law and ICAN -- when a pyrophosphoric acid is generated by coincidence with nucleic-acid magnification, such as law, without it uses a harmful reagent by using detection and the quantum approach of the pyrophosphoric acid of this invention -- high sensitivity -- and it became practical about the nucleic acid detection and to carry out a quantum simple.

[0065] Hereafter, when the PCR method is used as a nucleic-acid amplifying method, detection and the quantum approach of a nucleic acid of having used detection and the quantum approach of the pyrophosphoric acid of this invention are explained in detail.

[0066] An PCR reaction reacts as follows and separates considering a pyrophosphoric acid as a by-product. Therefore, the amount of pyrophosphoric-acid (PPi) generation is proportional to the amount of magnification DNA.

[0067]

[Formula 6]

$$(DNA)_n + dNTP \xrightarrow{\text{DNAポリメラーゼ}} (DNA)_{n+1} + PPi$$

(n＝DNA塩基数)

[0068] When carrying out the quantum of the amounts of initial mold nucleic acids, such as a virus, by this invention, it uses that a pyrophosphoric acid generates exponentially by PCR. When an PCR resultant shows an exponential increment mostly in early stages of a reaction and the sufficient number PCR of cycles is performed, the PCR resultant of the related almost same amount as the amount of initial mold nucleic acids is acquired. Therefore, the number of cycles (CT) which reaches a constant rate with an PCR resultant has the amount of initial mold nucleic acids, and the relation of inverse relationship. Moreover, in order to realize this relation, even if the amounts of initial mold nucleic acids differ, the amplification factor of a characteristic magnification term must be equal. Since this relation is materialized when a target sequence is the same, it is possible to presume the amount of initial mold nucleic acids from CT. In this invention, the number of cycles is changed, an PCR reaction is performed, and the approach of carrying out the quantum of the amount of initial mold nucleic acids from the amount of generation of the pyrophosphoric acid in each number of cycles is used.

[0069] In the same sample, the number of cycles is changed in the range of one to 40 cycle, and an PCR reaction is performed, and since the process which detects the amount of isolation pyrophosphoric acids

of each sample is included, it is carried out to the quantum of the amount of initial mold nucleic acids by the automated desirable continuous approach.

[0070] Therefore, detection and the quantum approach of the nucleic acid by this invention The 1st process of the heat denaturation which heats the sample solution containing a nucleic acid and makes a nucleic acid a single strand, The 2nd process which makes a priming nucleic acid generate by carrying out annealing of the oligonucleotide primer which has a complementary array to at least one specific base sequence in the single strand nucleic acid obtained at said 1st process, While making four sorts of deoxyribonucleoside triphosphoric acid, and DNA polymerase act on the priming nucleic acid generated at said 2nd process, carrying out an expanding reaction and compounding a DNA complementary strand The pyrophosphoric acid which separated at the process of the 3rd expanding reaction which separates a pyrophosphoric acid, and said 3rd process When detection and the 4th process which carries out a quantum are provided using any one approach among detection and the quantum approach of the five above-mentioned nucleic acids and the sample solution returns Said 1st, 2nd, 3rd, and 4th processes carry out multiple-times circulation one by one, and are performed (it is also hereafter called a "sample-ring current method".). Things are desirable.

[0071] Furthermore, detection and the quantum approach of the nucleic acid by this invention The expanding reaction in said 3rd process is performed in the reactor which fixed DNA polymerase. If detection and the quantum approach of the nucleic acid it is desirable that conversion of the pyrophosphoric acid in said 4th process is performed in the reactor which fixed the enzyme, and according to this this invention are used It was also found out by this invention person etc. that it becomes possible effectively about a nucleic acid detection and to carry out a quantum using the principle of the luciferin luciferase method which is a conventional method.

[0072] That is, in detection and the quantum approach of the nucleic acid by this invention mentioned above, an adenosine-triphosphate sulfurylase and luciferase are used as an enzyme fixed by the reactor with which conversion of a pyrophosphoric acid is performed in said 4th process, using the solution which added adenylyl sulfate and luciferin as a reagent for carrying out the quantum of the pyrophosphoric acid to the solution containing a pyrophosphoric acid as the sample solution. And a pyrophosphoric acid is changed according to an operation of an enzyme, and light is carried out detection and a quantum as matter to generate. According to the sample-ring current method of this invention, it becomes detectable [a momentary light] and a nucleic acid can be carried out detection and a quantum.

[0073] Although measurement of the amount of initial mold nucleic acids using detection and the quantum approach of the pyrophosphoric acid of this invention can be carried out using the temperature-circuit system which is equipment for the conventional PCR reaction if the case where the PCR method is used as a nucleic-acid amplifying method is taken for an example

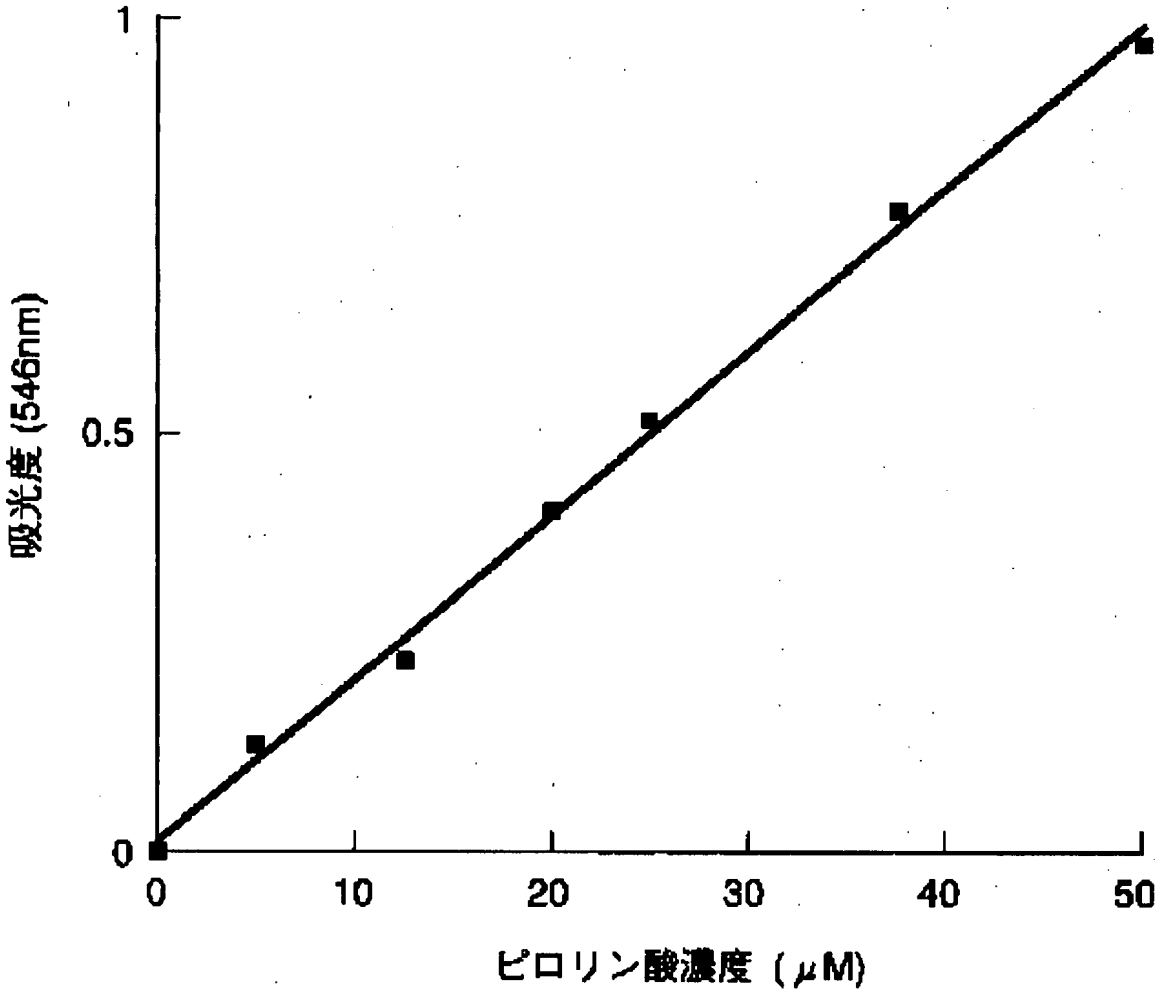
* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] Drawing showing the relation of the pyrophosphoric-acid concentration and the absorbance which were obtained using the pyrophosphoric-acid quantum approach of this invention.
[Drawing 2] Drawing showing the relation of the number of cycles and absorbance which were obtained using the nucleic-acid quantum approach of this invention.
[Drawing 3] The mimetic diagram showing one gestalt of the nucleic-acid quantitative analyzing instrument of this invention.
[Description of Notations]
1 12 [... The heating-at-high-temperature section, 5 / ... A low-temperature heating unit, 6 / ... 7 A moderate temperature heating unit, 9 / ... A reactor, 8 / ... The enzyme reaction section, 10 / ... A light absorption detector, 11 / ... A personal computer, 12 / ... Preparative isolation container] ... A container, 2 ... A liquid-sending pump, 3 ... A sample inlet, 4

[Translation done.]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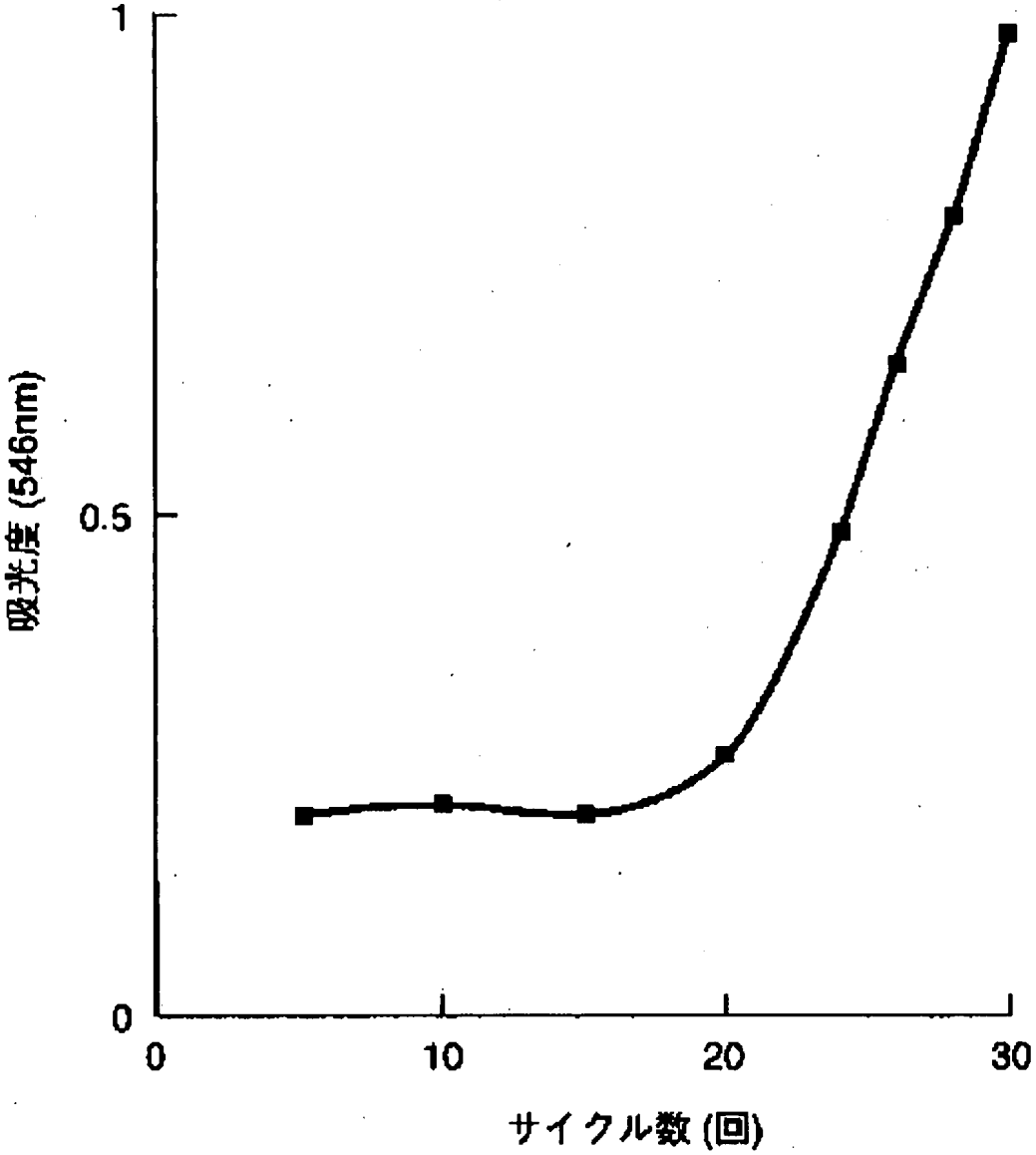
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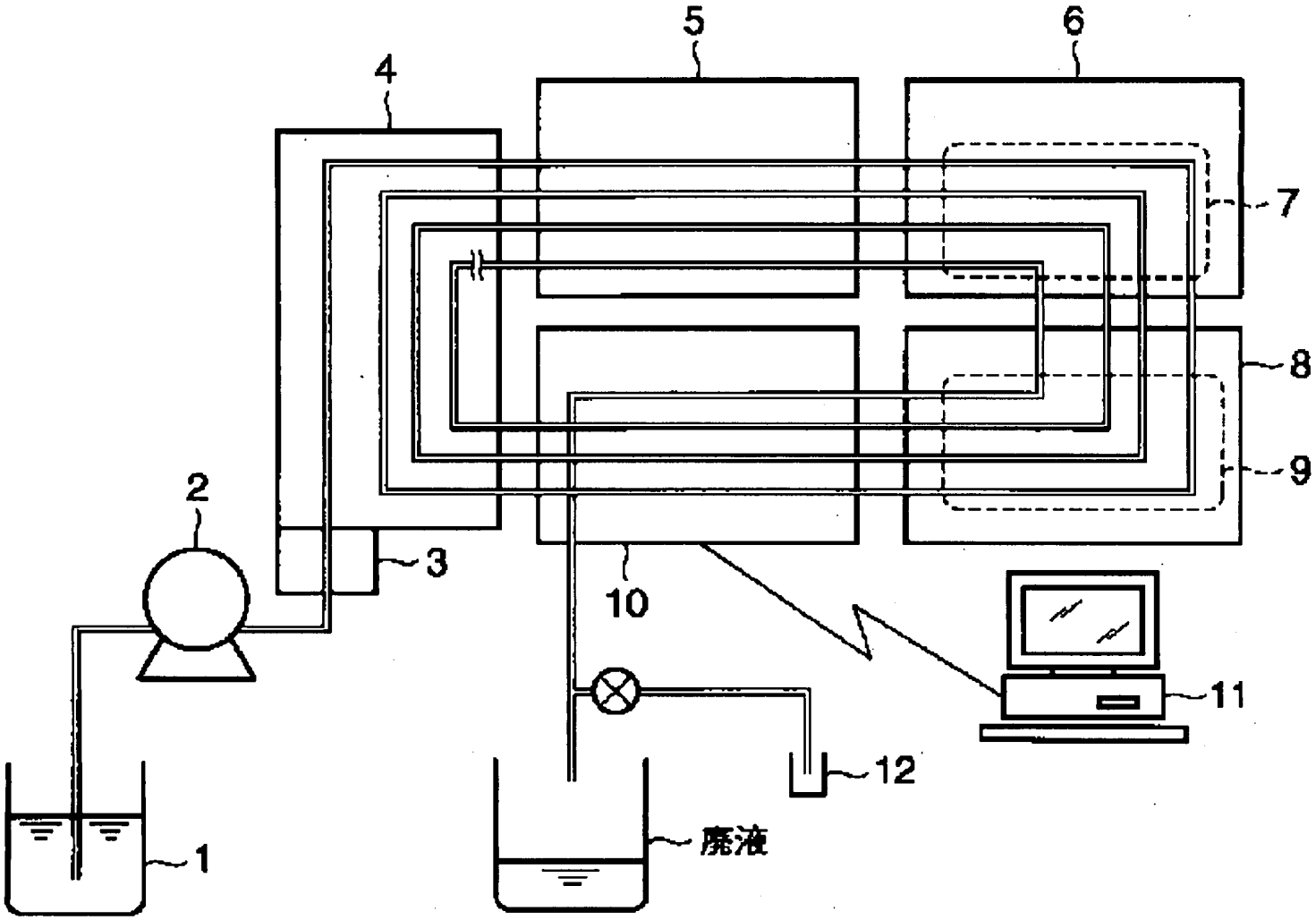
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